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Article

Biological screening of herbal extracts and essential oil from *Plectranthus* species: α-amylase and 5-lipoxygenase inhibition and antioxidant and anti-*Candida* potentials

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The phenolic compound content, the antioxidant and α-amylase inhibition potentials of different extracts of the *Plectranthus amboinicus*, *P. barbatus* and *P. ornatus* were evaluated. We also evaluated the influence of plant growth and harvest time on the chemical composition of the essential oil (EO) of *P. amboinicus*, its antioxidant and anti-*Candida* activities and the α-amylase and lipoxygenase inhibitions. The turbo-extract of *P. barbatus* showed the greatest phenolic compound content and antioxidant activity. No α-amylase inhibition activity was observed in the analyzed extracts, but the turbo-extraction and refluxing extracts possessed high antioxidant activities. Protected cultivation and morning harvest conditions gave the best antioxidant activities, which was associated to the highest carvacrol content. *P. amboinicu*s EO antioxidant activity could contribute to the reduction of oxidative stress in diabetes. Causal *Candida* strains of diabetic foot ulcers showed sensitivity to *P. amboinicus* EO. *C. albicans* and *C. dubliniensis* were the most sensitive of the selected *Candida* strains. Turbo-extracts or refluxing of the three species extracts and the EO of *P. amboinicus* should be considered as a potential candidate for the management the complications of type 2 diabetes.

Keywords: *Plectranthus amboinicus*. *Plectranthus barbatus*. *Plectranthus ornatus*. Extraction methods. Biological activities.

INTRODUCTION

Species such as *Plectranthus amboinicus* (Lour) Spreng, *Plectranthus barbatus* Andr., and *Plectranthus ornatus* Codd., belongs to the Lamiaceae family, exhibit different chemical and pharmacological properties. Their pharmacological properties have been frequently attributed to the presence of bioactive volatile and nonvolatile compounds belonging to different classes of phytochemicals, such as monoterpenoids, sesquiterpenoids, diterpenoids and phenolic compounds (Arumugam, Swamy, Sinniah, 2016; Brito *et al*., 2018).

In folk medicine, *Plectranthus* species leaves are commonly used as medicinal plants to treat inflammation-related diseases, particularly skin, infection, digestive, and respiratory problems (Saad *et al*., 2017). Extracts as well as essential oil from *Plectranthus* leaves have been explored for medicinal purpose (Brito *et al*., 2018; El-Hawary *et al.,* 2013; Falé *et al*., 2009; Mota *et al*., 2014; Vera, Mondon, Pieribattesti, 1993). The most common ethnobotanical uses of *P. barbatus* and *P. ornatus* are in the treatment of digestive conditions (Brito *et al*., 2018; Saad *et al*., 2017). Some studies have been shown that the

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presence of labdane type diterpenes present in leaves extracts of *P. barbatus* and *P. ornatus* are responsible for gastric secretion and explains why these plants are widely used in digestive disorders (Alasbahi, Melzig, 2010; Lakshmanan, Manikandan, 2015; Mesquita *et al*., 2021). *P. barbatus* is a world-renowned medicinal plant used in a form of infusion or decoction to treat liver and stomach disorders, while *P. ornatus* is indicated to alleviate gastritis, heartburn, upset stomach, and hangover (Falé *et al*., 2009; Brito *et al*., 2018; Mesquita *et al*., 2021). Although, *P. amboinicus* is also reported used in dysentery and digestive disorders, its mainly used to treat infectious and dermatological conditions (Lakshmanan, Manikandan, 2015; Arumugam, Swamy, Sinniah, 2016). According Arumugam, Swamy and Sinniah (2016), *P. amboinicus* essential oil and its various solvent extracts are used for skin ulcerations, skin allergies, and applied to cuts or burns, acting as an antiseptic and promote healing. At the Medicinal Garden of Federal University of Lavras (UFLA), many people seek *P. barbatus* and *P. ornatus* for the treatment of gastrointestinal disorders and *P. amboinicus* to treat onycomycosis and throat infections.

The great diversity of plant species not yet studied represents a vast field of molecules to be discovered for therapeutic purpose (Pereira *et al*., 2011). Diabetes mellitus type 2 constitutes a very important public health problem worldwide due to the increase in cases and the severity of the associated complications (Imbert *et al*., 2016). In the last decade, some investigations about the use of *Plectranthus* species for the control or treatment of the complications of diabetes mellitus type 2 have been published, but they are still underexplored (Dhakshinya *et al*., 2019; Koti *et al*., 2011).

Amylase inhibitor phytochemicals offer a promising strategy for the control of diabetes-associated hyperglycemia type 2, and also obesity and hypertension preventions by decreasing the breakdown of starch, reducing hyperglycemia (Pereira *et al*., 2011; Fatima *et al*., 2019; Sharma *et al*., 2020). The lipoxygenase enzyme is involved in diabetes complications, such as vein inflammation, circulatory injury and endothelial cell damage and hemodynamic imbalance caused by atherosclerosis (Domingueti *et al*., 2016; Dobrian *et al*., 2019; Dong *et al*., 2020). Another complication of diabetes is the prevalence of mycosis in the feet of patients with diabetes, which may favor the development of diabetic foot. Onychomycosis in patients with diabetes increases the possibility of developing foot ulcers of bacterial or yeast-form etiology, which may end in amputation. In this case, the most common etiological agents are *Candida* species. Furthermore, in diabetes mellitus, increased oxidative stress and lipid peroxidation contribute to chronic complications (Imbert *et al*., 2016; Kumar *et al*., 2016; Rodrigues, Rodrigues, Henriques, 2019).

Despite the consideration that biological activities may change depending on genetic and environmental conditions, we first reported an exploratory study of the phenolic compound content and the potential antioxidant and α-amylase inhibition activities of different extracts of three species of the *Plectranthus* genus (*P. amboinicus*, *P. barbatus* and *P. ornatus*). In a second step, considering that chemical profiles from the same species often show differences depending on environmental conditions and agricultural practices, we evaluated the influence of the growing and harvest time conditions on the essential oil (EO) of *P. amboinicus* and its antioxidant, α-amylase and lipoxygenase inhibition and anti-*Candida* activities.

MATERIAL AND METHODS

Phenolic compound content, antioxidant activity and α-amylase inhibition

Three *Plectranthus* species were grown in beds during 240 days (21º 14' S e 45º 00 W, at 918 m altitude). They were fertilized with cattle manure in 3.0 kg m^{-2} dose and irrigated four times a week. Exsiccates were deposited in the Herbarium of the Minas Gerais Agricultural Research Company (PAMG/EPAMIG) under vouchers PAMG 57803 (*P. barbatus*), PAMG 57804 (*P. amboinicus*) and PAMG 57805 (*P. ornatus*).

The experiment was performed in a completely randomized design according to a 3×3 factorial scheme, with three species and three extraction methods. The extracts (5% w/v) were prepared by refluxing, infusion, or turbo-extraction from fresh leaves of *P. barbatus*, *P. amboinicus,* and *P. ornatus*. Water was the solvent for the extracts obtained by refluxing and infusion, while a 70%

ethanol solution was utilized for the turbo-extraction. The refluxing extract was distilled for 30 min. The infusion was prepared by pouring distilled water at 80°C over the plant material and subjecting it to static maceration for 10 min in a capped container. For the turbo-extraction, each species was extracted with 3 cycles of 10 min in an ice bath with an interval of 1 min between them. After the extractive procedures, the aqueous and hydroalcoholic extracts were filtered and kept in a freezer at -20°C until analysis. To obtain the extractives yields, 25 mL of each extract was evaporated under low pressure to dryness. The dry residue was determined according to the Brazilian Pharmacopoeia V (Brasil, 2010).

The amounts of total phenolic compounds (TPCs), total flavones/flavonols (TFFs), and total flavanones/dihydroflavonols (TFDs) of the extracts were determined. The measurement of TPCs was conducted according to the Folin-Ciocalteu method (Slinkard, Singleton, 1977). The concentration of the calibration curve ranged from 1.27 to 0.009 mg/mL in an ethanol solution of gallic acid and the TPC content of the extracts was expressed in milligrams of gallic acid equivalents per gram of fresh leaf (mg GA/g). The TFFs were determined by the method of Ahn *et al*. (2007). The concentration of the calibration curve ranged from 1.71 to 0.013 mg/mL in an ethanol solution containing quercetin and the TFF content of the extract was expressed in milligrams of quercetin equivalents per gram of fresh leaf (mg QE/g). Quantification of TFDs was determined by the method of Popova *et al*. (2004). The calibration curve comprised concentration range from 2.0 to 0.015 mg/mL in a methanolic solution of naringenin and the TFD content in the extracts was expressed in milligrams of naringenin equivalents per gram of fresh leaf (mg NE/g).

The antioxidant capacities of the extracts were evaluated using five different methods that are described as follows: the total antioxidant capacity (TAC) was measured based on the method of reduction of ammonium molybdate described by Prieto, Pineda, Aguilar (1999). The calibration curve of ascorbic acid ($y = 3.4675x + 0.1722$, $R^2 = 0.9951$), used to determine the activity, comprised concentration range from 0.65 to 0.010 mg/mL. The results were expressed in milligrams of ascorbic acid equivalent per gram of fresh leaf (mg AA/g). The scavenging activity of DPPH (1,1-diphenyl-1,2-picrylhydrazyl) was determined based on the method proposed by Brand-Williams, Cuvelier, Berset (1995). BHT was used as a positive control ($IC_{50\%}$= 0.1826 ± 0.0136 mg/mL) and the calibration curve comprised concentration range from 10.0 to 0.078 mg/mL. The radical scavenging activity of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was carried out using the method described by Re *et al*. (1999), with minor modifications. Briefly, the ABTS radical was generated by reacting an aqueous solution of $K_2S_2O_8$ (2.45 mM) in the dark for 16 h at room temperature to obtain an absorbance of 0.700 at 734 nm. Then, 30 µL of the extracts were added to 270 µL of ABTS, and the absorbance was read at 734 nm after 6 minutes. Trolox was used as a positive control ($IC_{50\%}$= 0.0074 ± 0.0001 mg/mL) and the calibration curve comprised concentration range from 0.2 to 0.001 mg/mL. The ferrous ion chelating power (CP) was determined according to Miguel (2010). EDTA was used as a positive control ($IC_{50\%}$= 0.04 ± 0.01 mg/mL) and the calibration curve comprised concentration range from 2.5 to 0.039 mg/mL. The method described by Aazza *et al*. (2014) was utilized to determine the liposome peroxidation inhibition (LPI) of the extracts. BHT was used as a positive control and the calibration curve comprised concentration range from 10.0 to 0.078 mg/mL. The results of DPPH, ABTS, CP and LPI were expressed as IC_{50} (mg/mL) values.

The inhibition assay of α-amylase from porcine pancreas (EC 3.2.1.1 – Sigma-Aldrich®) was performed according to Xiao, Storms, Tsang (2006) table based on the starch-iodine test. Phaseolamine was used as a positive control ($IC_{50\%}$= 0.0067 ± 0.0016 mg/mL) and the calibration curve comprised concentration range from 0.57 to 0.017 mg/mL. Inhibition activity was expressed as the IC_{50} (mg/mL).

Effect of growing and harvest time on EO of *P. amboinicus* and its α-amylase and lipoxygenase inhibition and antioxidant and anti-*Candida* activities

The experiment was conducted in a completely randomized design according to a 2×2 factorial scheme comprising two types of cultivation (field and protected)

and two harvest times (9:00 am and 1:00 pm), with plants that were 240 days of age and four replicates (five plants for each replicate). The scions of *P. amboinicus* were produced through micro cuttings (±5 cm) using apical buds from mother plants. After rooting, plants were transplanted into pots of a 5 L capacity (protected cultivation) and into beds that were 6 × 1.20 m^2 (field cultivation). The soil of the bed was the same as that used in the pots, and all plants were irrigated periodically 4 times a week.

For EO distillation, 200 g of *P. amboinicus* fresh leaves was hydrodistilled with 1 L of water in a modified Clevenger apparatus for 120 min in quadruplicate. The EO were separated through decanting and removed from the distiller tube by means of a micropipette. The samples were stored in a freezer at -10 °C until chemical analyses. The moisture content of fresh leaves was determined using an infrared moisture analyzer OHAUS MB45 set to 105 °C for 5 min. The EO content was determined and expressed in g/100 g of leaf dry matter.

The EO chemical analyses were carried out by gas chromatographic techniques using the same apparatus but with some modifications in the operation conditions described by Bibiano *et al*. (2019). The modifications of Bibiano's method comprised the injector temperature (set at 240 °C) and the following temperature ramp: the initial oven temperature was 50 °C, followed by a 3 °C/min temperature increase to 180 °C, then an increase of 10 °C/min to 280 °C, and a final isothermal period of 1 min.

To determine the antioxidant capacity (TAC, DPPH and ABTS) and the potential of α-amylase and 5-lipoxygenase inhibition of *P. amboinicus*, 25 µL of EO at dilutions between of 1/50 and 1/1600 were combined with the reagent solutions of each assay. For TAC, 25 µL of the EO, in the dilution that fitted at midpoint of the calibration curve (dilution 1/100), was mixed with 1000 µL of the reagent solution. The calibration curve of ascorbic acid comprised the range of 0.19 to 2.2 mg/mL ($y = 0.2219 x + 0.1417$, $R^2 = 0.9992$). The radical scavenging activity of DPPH and ABTS were determined by adding 275 µL of their respective solutions to 25 µL of the oil (dilution 1/50 to 1/1600). The data were expressed as IC_{50} (mg/mL) values.

For enzymatic inhibition assays, over 50 µL of the EO solution at 1/10, 1/50, 1/100 and 1/200 dilutions were added to the reagents. The α-amylase inhibition potential of the *P. amboinicus* EO was determined in the same way as the extracts. The 5-lipoxygenase (EC 1.13.11.12) assay was performed according to the procedure described by Boulanouar *et al*. (2013). Nordihydroguaiaretic acid (NDGA) was used as a positive control ($IC_{50\%}$= 0.0200 ± 0.0030 mg/mL). The inhibition activity was expressed as the IC_{50} (mg/mL).

The anti-*Candida* activity of the EO of *P. amboinicus* was evaluated using a blended sample prepared with equivolumetric aliquots of each sample: protected cultivation and morning harvest (PCM), protected cultivation and afternoon harvest (PCA), field cultivation and morning harvest (FCM), field cultivation and afternoon harvest (FCA). *Candida* species used in this study were reference strains and selected according to Clinical and Laboratory Standards Institute (CLSI) quality criteria (CLSI, 2009). The determination of the sensitivity of the EO was performed by the disc-diffusion technique in agar as described in document M44-A2 of CLSI (CLSI, 2009). The tested microorganisms were *Candida rugosa* (IZ-12), *C. kruseii* (ATCC 6258), *C. tropicalis* (CBS 94), *C. dubliniensis* (CBS7987), *C. albicans* (ATCC 90028), *C. utilis* (CBS 5609), *C. kruseii* (CBS 572), *C. lusitanea* (IZ-06), *C. gablata* (IZ-07), *C. gablata* (ATCC 5207), and *C. albicans* (CBS 562). The Candida strain were provided by the Laboratories of Pharmacogenetics and Molecular Biology at José do Rosário Vellano University/UNIFENAS and by the Laboratory of Microbiology and Immunology at Piracicaba Dentistry University/UNICAMP. The yeast suspension was prepared in physiological saline (0.85% NaCl) with a turbidity equivalent to the 0.5 McFarland scale standard (106 cells/mL) and spread with a sterile swab on the surface of Müeller-Hinton agar (supplemented with 2% glucose and 0.5 µg/mL methylene blue). Disks containing 5 µL of the oils were applied to the surface of the agar and incubated at 35°C. After 18 to 24 h, the growth inhibition halo was measured.

Statistical analysis

All assays were performed in triplicate. Sisvar software version 5.3. was used for analysis of variance by the F test and the average between the treatments

compared by the Scott-Knott test ($p \leq 0.05$). Statistica® software version 13.3 was used for principal component analysis (PCA).

RESULTS AND DISCUSSION

Phenolic compound content, antioxidant activity and inhibition of α-amylase

The extraction yields of the three species were dependent on the plant and the extraction method (Table I). The highest extraction yield was found for alcoholic turbo-extraction of *P. barbatus* (5.61%) and for refluxing of *P. amboinicus* (4.72%) and *P. ornatus* (5.34%). The infusion method exhibited the lowest yield, independent of the *Plectranthus* species (1.20 to 1.75%).

Factors such as the natural variability of the herbal material, combined with the solvent system, extraction method and extraction conditions, can all have a significant impact on the quantity and composition of an herbal extract (TGA, 2011). Aqueous infusions and decoctions are the most traditional oral forms used in folk medicine, popularly named tea. In addition to aqueous extracts, alcoholic extracts are also widely used in herbal medicine preparations. To compare the extraction method on the phenolic compound content and biological activities, we prepared traditional aqueous forms (infusion and refluxing) and alcoholic turbo-extraction *Plectranthus* extracts.

The total phenolic compound (TPC), total flavone/flavonol(TFF) and the total flavanone/dihydroflavonol (TFD) contents among the different extracts are presented in Table I. The TPC, TFF, and TFD of each species were influenced by the extraction method utilized. Refluxing enabled greater extraction of TPCs from *P. amboinicus* (9.31 mg GA/g) and *P. ornatus* (9.20 mg GA/g). However, extracts from alcoholic turbo-extraction of *P. barbatus* showed the highest phenolic contents (11.67 mg GA/g), although it was quite similar to that of the aqueous reflux extract. Turbo-extraction proved to be the most effective method of TFF extraction for *P. ornatus* (8.23 mg QE/g), *P. barbatus* (2.50 mg QE/g), and *P. amboinicus* (0.89 mg QE/g). Similarly, turbo-extraction resulted in the relatively high extraction of TFDs from *P. barbatus* (7.82 mg NE/g) and *P. ornatus* (6.61 mg NE/g), while the highest TFD content in *P. amboinicus* (3.20 mg NE/g) was found in the aqueous reflux extract.

TABLE I - Total phenolic and flavonoids contents, and antioxidant activities of different extracts of three *Plectranthus* species

	Extractive yields (%)			TPC[a] (mg GA/g)			TFF[b] (mg QE/g)			TFD[c] (mg NE/g)		
	PA	**PO**	**PB**	**PA**	**PO**	**PB**	**PA**	**PO**	**PB**	**PA**	**PO**	**PB**
Refluxing	4.72 Ab	5.34 Aa	3.69 Bc	9.31 Ab	9.20 Ab	10.80 Ba	0.60 Bc	0.90 Cb	2.35 Ba	3.20 Ab	2.70 Bc	3.58 Ba
Infusion	1.20 Cb	1.75 Ca	1.71 Ca	1.46 Cb	1.57 Cb	3.37 Ca	0.08 Cc	1.57 Ba	0.32 Cb	1.07 Ca	1.10 Ca	0.85 Cb
Turbo extraction	3.63 Bc	4.80 Bb	5.61 Aa	7.56 Bc	8.23 Bb	11.67 Aa	0.89 Ac	8.23 Aa	2.50 Ab	3.02 Bc	6.61 Ab	7.82 Aa
	TAC[d] (mg AA/g)			**DPPH (IC_{50}=mg/mL)**			**ABTS (IC_{50}=mg/mL)**			**CP[e] (IC_{50}=mg/mL)**		
	PA	PO	PB	PA	PO	PB	PA	PO	PB	PA	PO	PB
Refluxing	1.51 Ac	2.12 Ab	2.70 Ba	0.51 Ca	0.50 Ca	0.28 Bb	0.75 Ca	0.72 Ca	0.45 Bb	5.19 Ca	3.81 Cc	5.08 Bb
Infusion	0.19 Ca	0.03 Cb	0.21 Ca	4.47 Ab	4.68 Aa	2.07 Ac	4.50 Aa	1.96 Ac	3.74 Ab	15.78 Aa	15.61 Ab	14.16 Ac

TABLE I - Total phenolic and flavonoids contents, and antioxidant activities of different extracts of three *Plectranthus* species

	Extractive yields (%)			TPC[a] (mg GA/g)			TFF[b] (mg QE/g)			TFD[c] (mg NE/g)		
Turbo extraction	1.16 Bc	1.50 Bb	2.96 Aa	0.75 Ba	0.68 Bb	0.12 Cc	0.98 Ba	0.97 Ba	0.33 Cb	8.56 Ba	5.18 Bb	3.04 Cc

Averages followed by the same lowercase letter on the rows and capital on the columns belong to the same group by Scott-Knott test at 5% probability. TPC[a]: Total phenolic compounds expressed in mg of gallic acid equivalents/g of fresh leaf (mg GA/g). TFF[b]: Total flavones and flavonols expressed in mg of quercetin equivalents/g of fresh leaf (mg QE/g). TFD[c]: Total flavanones and dihydroflavonols expressed in mg of naringenin equivalents/g of fresh leaf (mg NE/g). TAC[d]: Total antioxidant capacity expressed as mg of ascorbic acid equivalents/g of fresh leaf (mg AA/g). CP[e]: Chelating power. PA: *P. amboinicus.* PO: *P.ornatus.* PB: *P. barbatus.* DPPH (BHT): 0.18 ± 0.01 mg/mL. ABTS (Trolox): 0.07 ± 0.01 mg/mL. CP (EDTA): 0.04 ± 0.01 mg/mL.

The three species of *Plectranthus* had higher levels of TFD than of TFF. The highest TFF contents of all species were obtained with alcoholic turbo-extraction. The TFD content varied between species and extraction methods. *Plectranthus amboinicus* showed a higher TFD content with aqueous refluxing, whereas *P. ornatus* and *P. barbatus* obtained better results with alcoholic turbo-extraction.

To understand the influence of extraction method on phenolic compounds, principal component analysis (PCA) was used to distinguish TPCs, TFFs, and TFDs occurring in *P. amboinicus*, *P. barbatus* and *P. ornatus* (Figure 1). The resulting PCA scores and loadings provide a conceptual overview of the treatments by showing a total of 95.96% of the variance. The analyses of scores divided the treatments into two groups, where it was possible to observe that infusion proved to be the least effective method for the extraction of phenolic compounds and, overall, turbo-extraction was the best method. According to Oh *et al*. (2013), in general, ethanol extracts contain more phenolic compounds than those contained in aqueous extracts. However, in the present study, the aqueous refluxing method was also able to recover high amounts of TPCs in the *Plectranthus* species.

The great difference in TPC, TFF and TFD contents between the aqueous infusion and refluxing extracts showed that extraction temperature had a significant impact on phenolic compound recoveries. Falé *et al*. (2009) evaluated the extraction yields of decoctions and infusions of five species of *Plectranthus* and noted variations between species and the extraction method. The results of Falé *et al*. (2009) for *P. barbatus* corroborate those of the present study, in which the contact of the plant material with a high temperature is an important parameter for achieving greater extraction yields in these species (Table I).

The total antioxidant capacity (TAC) was dependent on the species and extract preparation. *P. barbatus* showed the highest TAC. The extracts obtained by aqueous refluxing of *P. amboinicus* (1.51 mg AA/g) and *P. ornatus* (2.12 mg AA/g) and the extract by alcoholic turbo-extraction of *P. barbatus* (2.96 mg AA/g) showed the highest TAC values (Table I). In alcoholic turbo-extraction, the TAC exhibited by *P. barbatus* was 1.97-fold higher than that of the *P. ornatus* and 2.55-fold higher than that of the *P. amboinicus* extracts.

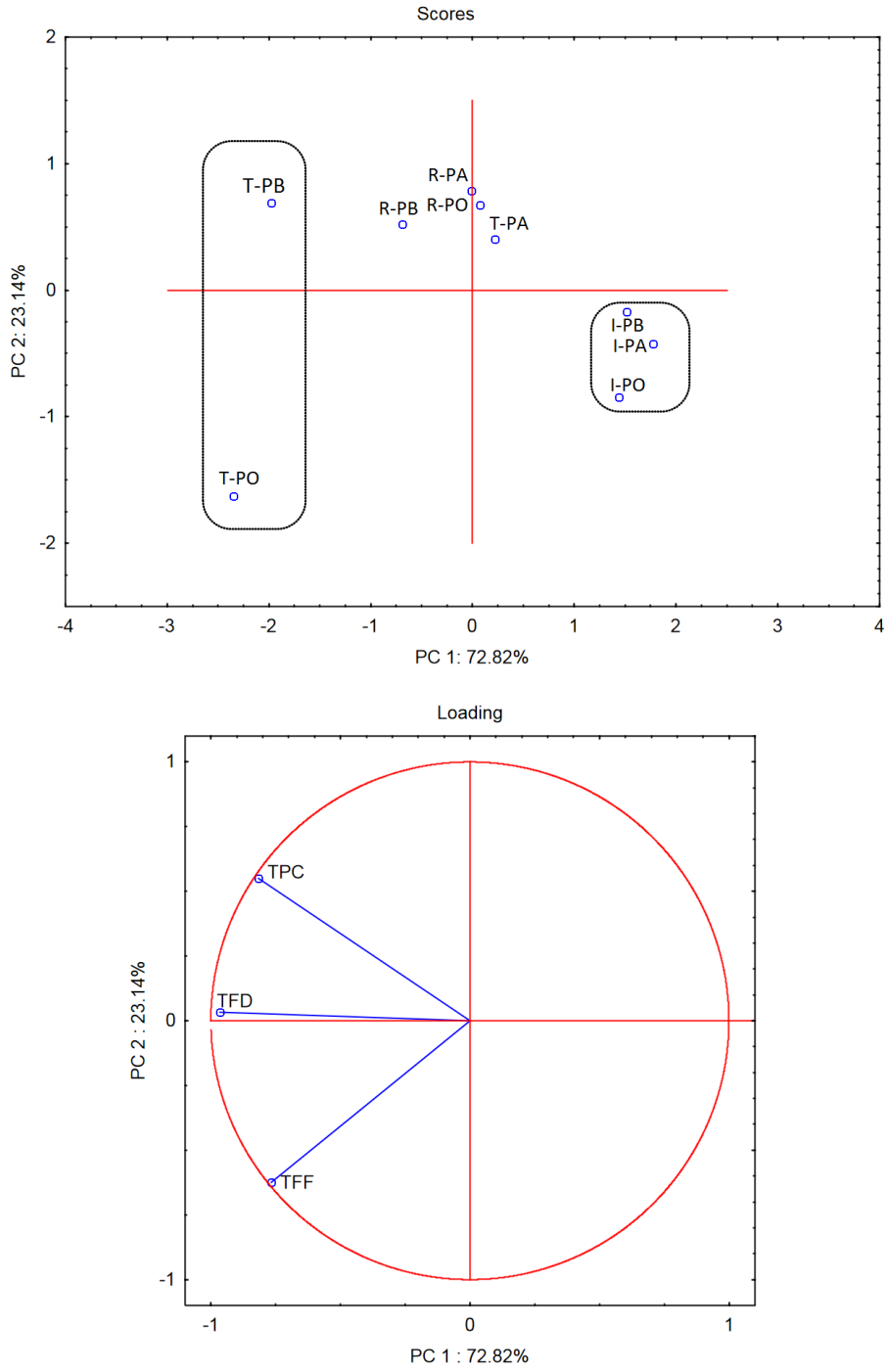

FIGURE 1 - Principal component analysis on the matrix correlation built using data of the total phenolic (TPC), flavones/flavonols (TFF) and flavanone/dihydroflavonol (TFD) contents for three species of *Plectranthus amboinicus* (PA), *P. ornatus* (PO) and *P. barbatus* (PB) in three extraction methods turbo-extraction (T), refluxing (R), and infusion (I).

The DPPH and ABTS assays are the most common, easy, and simple methods for estimating the scavenging ability of free radicals (Niveditha, Sridhar, 2014). The DPPH assay is based on the decrease in the absorbance at 515 nm of the DPPH$^{\bullet}$ solution due to the inactivation of the DPPH$^{\bullet}$ radicals from the antioxidants present in the sample (Niveditha, Sridhar, 2014). The blue/green ABTS$^{\bullet+}$ chromophore is produced through the reaction

between ABTS and potassium persulfate (Re *et al.*, 1999). The antioxidants were determined by the decolorization of $ABTS^{\bullet+}$ by measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm.

Among all extracts, the IC_{50} varied from 0.12 to 4.68 mg/mL for DPPH scavenging and 0.33 to 4.50 mg/mL for ABTS scavenging (Table I). Similar to the TAC results, *P. barbatus* presented the best antioxidant activities by free radical scavenging of DPPH and ABTS radicals, which was nearly three times higher than that of the two other species. Comparing the alcoholic turbo-extracts of *P. barbatus* and *P. ornatus*, the former was approximately 5.66 and 2.93 times more active than the latter in DPPH and ABTS free radical scavenging, respectively. The antioxidant activity of the alcoholic turbo-extracts of *P. barbatus* (IC_{50} = 0.12 mg/mL) with regard to their ability to scavenge DPPH free radicals was high compared to that of BHT (IC_{50} = 0.18 mg/mL), which was used as a standard. Falé *et al*. (2009) also demonstrated that decoctions of *P. barbatus* exhibited high antioxidant activity according to the DPPH test (IC_{50} = 45.8 ± 0.51 µg dry extract/mL).

Several authors have attributed the increased antioxidant activity of natural products to the presence of high levels of phenolics (Ahn *et al.*, 2007; Falé *et al.*, 2009; Gülçin *et al.*, 2010). Phytochemical screening studies of extracts of the species *Plectranthus* have shown the presence of different constituents, such as flavonoids, acids, esters, phenolics, phenylpropanoids, and diterpenes, which may contribute to antioxidant activities (Falé *et al.*, 2009). Among the phenolic constituents, rosmarinic acid has been associated with digestion-related ethno-uses of *P. barbatus* decoctions (Brito *et al.*, 2018). In addition, other components of nonphenolic nature, such diterpenes, might also partially explain the antioxidant activity of *Plectranthus* spp. (Kabouche *et al.*, 2007).

Chelating agents form σ-bonds with metal ions and act as secondary antioxidants by reducing the redox potential of metal ions. The interaction of polyphenols with transition metal ions can also underlie their pro-oxidant effect. Flavonoids can reduce metal ions, which promote the Fenton reaction (Niveditha, Sridhar, 2014). Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. Chelation may afford protection against oxidative damage by removing iron (Fe^{+2}) that may otherwise participate in HO**.**-generating, Fenton-type reactions (Gülçin *et al.*, 2010). The mean values obtained in the assay of the chelating power (CP) of the extracts of *P. amboinicus*, *P. ornatus*, and *P. barbatus* are shown in Table I. The best CP was obtained for alcoholic turbo-extracts of *P. barbatus* (3.04 mg/mL), which could be comparable to the power of Fe^{+2} ion chelation of the aqueous reflux extract of *P. ornatus* (3.81 mg/mL). The lowest chelating activity was observed in the aqueous infusion extracts of the three species (14.16 to 15.78 mg/mL).

PCA was applied to the antioxidant activities (TAC, DPPH, ABTS and CP) and *P. amboinicus*, *P. ornatus* and *P. barbatus* (Figure 2). The two principal components (PC1+PC2) account for 95.17% of the data variance. It is possible to observe in the score plots that the data were separated into two groups: the infusion from the refluxing and turbo-extraction methods. PCA confirmed that the alcoholic turbo-extract had the same antioxidant activity as that of the aqueous refluxing extract (Figure 2). The refluxing method is quite similar to the decoction method. The only difference between them is that refluxing is carried out in a closed system without water evaporation loss, and decoction is performed in an open system with water evaporation loss. Since the refluxing method is closely related to the decoction method, these findings indicated that the decoction is the best traditional form for *Plectranthus* ethno-preparation. PCA also confirmed that the infusion method resulted in extracts with low antioxidant activities.

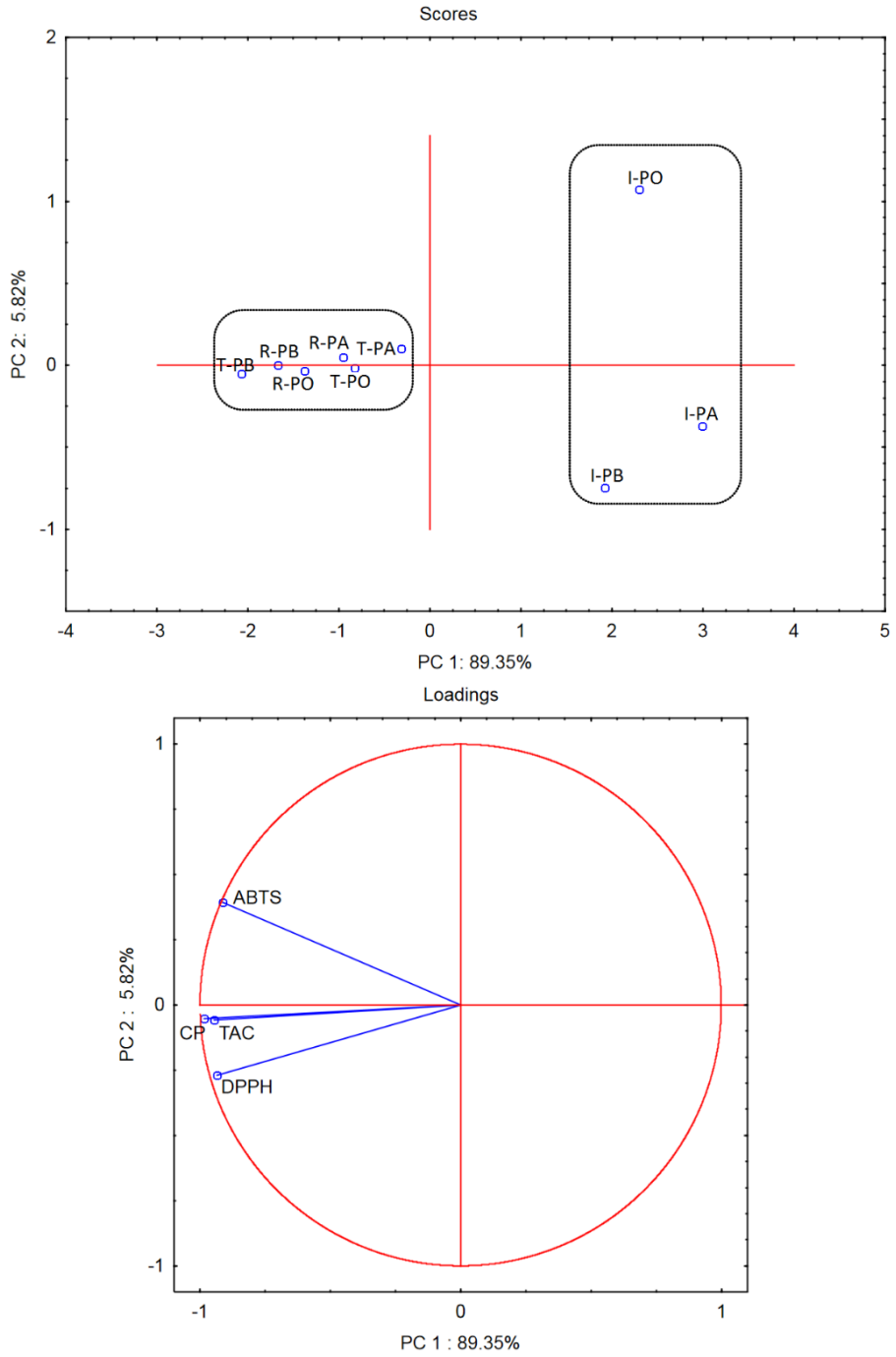

FIGURE 2 - Principal component analysis on the matrix correlation built using data of antioxidant activity (TAC), DPPH, ABTS and chelating power (CP) for three species of Plectranthus amboinicus (PA), P. ornatus (PO) and P. barabatus (PB) in three extraction methods turbo-extraction (T), refluxing (R), and infusion (I).

Koti *et al.* (2011) provided evidence that an ethanolic extract of *P. amboinicus* had antidiabetic activity mediated through the regulation of carbohydrate metabolic enzyme activities. Therefore, we evaluated the potential of all *Plectranthus* extracts to inhibit the α-amylase enzyme to investigate their potential in controlling type 2 diabetes. Phaseolamine was used as a standard to analyze the activity of α-amylase

inhibition, and the IC_{50} was calculated as 0.0067 ± 0.0016 mg/mL. For the studied concentrations, a 50% inhibition of the α-amylase enzyme was not observed for any *Plectranthus* extract. The extracts showed 11.37 to 45.67% inhibition of α-amylase. The maximum percentage was observed in the alcoholic turbo-extract of *P. barbatus*.

Recently, Dhakshinya *et al*. (2019) demonstrated the inhibitory activity against α-amylase and α-glucosidase of a fraction derived from a *P. amboinicus* methanolic extract. The maximum percentage of α-amylase and α-glucosidase enzyme inhibition was 75.68 ± 0.97 and 67.35 ± 1.10, respectively, at a concentration of 500 µg/mL in the isolated fraction. Therefore, we could not conclude that *P. amboinicus*, *P. ornatus* and *P. barbatus* do not have α-amylase inhibition activity since the fractionation of these crude extracts could ameliorate this activity. We concluded that the *P. amboinicus*, *P. ornatus* and *P. barbatus* preparations possessed antioxidant activities. According to Koti *et al*. (2011), high levels of oxidative cytotoxicity have been linked to glucose oxidation, lipid abnormalities and nonenzymatic glycation of proteins, which contribute to the development of diabetic complications. Phytochemical antioxidants might be an effective strategy for reducing diabetic complications due to disproportionate generation of free radicals. Thus, *P. amboinicus*, *P. ornatus* and *P. barbatus* preparations can be considered potential candidates for the management of type 2 diabetes complications.

Effect of growing and harvest time on EO of *P. amboinicus* and its antioxidant, α-amylase and lipoxygenase inhibition and anti-*Candida* activities

Hydrodistillation of the fresh leaves provided yellowish EO with a strong characteristic odor. The fresh leaves of *P. amboinicus* cultivated in the field and under protected cultivation conditions showed moisture contents of 43.09 ± 2.90 and 45.91 ± 2.45%, respectively. These values were used to express the EO content in g/100g dry leaves. The EO content in the leaves was 0.048% (morning) and 0.051% (afternoon) in protected cultivation and 0.100 (morning) and 0.103% (afternoon) in field cultivation. *P. amboinicus* plants grown in the field accumulated approximately 50% more EO than those grown under protected cultivation conditions. A similar EO content to that in our findings was observed by Vera, Mondon, Pieribattesti (1993), who found 0.07% hydrodistilled EO from fresh leaves and stems of *P. amboinicus* grown in France. However, a greater content was found by Bandeira *et al*. (2011) in the EO distilled from fresh leaves of *P. amboinicus* cultivated in southern Brazil (0.43%).

GC and GC-MS chemical analyses of *P. amboinicus* EO identified a maximum of sixteen chemical components, among which monoterpenes and sesquiterpenes accounted for more than 98.69% of the total chemical composition (Table II). Among these compounds, 69.07 to 75.21% comprised the total phenolic monoterpenes represented by thymol (0.13 to 0.16%) and carvacrol (68.92 to 75.21%). The type of cultivation and harvest time changed the qualitative and quantitative chemical composition of the *P. amboinicus* EO. In the plants grown under protected cultivation conditions and harvested in the morning, the presence of 1,8-cineole, thymol and caryophyllene oxide was not observed, and in field cultivation, α-pinene was not detected independently of harvest time. With respect to quantitative chemical composition, the greatest differences were observed for *o*-cymene and carvacrol. The lowest *o*-cymene content was observed in plants grown in the protected cultivation environment that were harvested in the morning (2.30%), and the highest *o*-cymene content was observed in the field-cultivated plants that were harvested in the afternoon (6.26%). A difference of approximately 9% was observed between the minimum and maximum carvacrol contents. The minimum accumulation of carvacrol content was observed in the field-cultivated plants that were harvested in the afternoon (68.92%), and the maximum was observed in the plants grown in protected cultivation conditions that were harvested in the morning (75.21%).

TABLE II - Chemical composition of essential oil of *P. amboinicus* under different growing and harvesting conditions

		Área % ± SD				
Compounds	**RI**	**PCM**	**PCA**	**FCM**	**FCA**	**Mix**
α-Pinene	932	0.05±0.00	0.08±0.00	nd	nd	0.05±0.00
Sabinene	969	0.56±0.00	0.27±0.00	0.22±0.00	0.32±0.00	0.52±0.00
β-Pinene	978	0.59±0.01	0.34±0.00	0.84±0.00	0.65±0.01	0.76±0.00
β-Myrcene	991	0.13±0.00	0.28±0.00	0.23±0.00	0.33±0.00	0.20±0.00
α-Terpinene	1015	0.31±0.01	0.51±0.00	0.39±0.00	0.47±0.00	0.45±0.01
o-Cymene	1022	2.30±0.07	4.35±0.05	4.84±0.03	6.26±0.03	5.30±0.02
1,8-Cineole	1026	nd	0.15±0.00	0.12±0.00	0.16±0.00	0.14±0.00
γ-Terpinene	1057	4.25±0.08	5.13±0.02	4.77±0.06	5.50±0.04	4.36±0.04
4-Terpineol	1174	1.32±0.01	1.31±0.02	1.24±0.00	1.18±0.00	1.12±0.01
Thymol	1287	nd	0.13±0.00	0.16±0.00	0.15±0.00	0.17±0.01
Carvacrol	1306	75.21±0.11	74.42±1.38	72.01±0.02	68.92±0.09	71.04±1.54
β-Caryophyllene	1415	7.70±0.04	6.68±0.12	6.81±0.02	7.18±0.04	7.53±0.05
α-Bergamotene	1434	3.84±0.03	3.49±0.06	3.17±0.01	3.65±0.01	3.41±0.03
α-Humulene	1449	2.04±0.01	1.81±0.03	1.86±0.00	1.91±0.01	1.89±0.00
Spathulenol	1577	0.75±0.01	0.90±0.01	1.76±0.01	1.83±0.01	1.72±0.02
Caryophyllene oxide	1603	nd	0.15±0.00	0.27±0.00	0.27±0.00	0.34±0.00
Monoterpene hydrocarbons		8.19	10.95	11.29	13.52	11.65
Oxygenated monoterpenes		1.32	1.46	1.37	1.34	1.26
Phenolic monoterpenes		75.21	74.55	72.17	69.07	71.21
Sesquiterpene hydrocarbons		13.57	11.39	11.83	12.73	12.82
Oxygenated sesquiterpenes		0.75	1.04	2.03	2.10	2.06
TOTAL (%)		99.04	99.39	98.69	98.77	98.99

RI: Retention index relative to *n*-alkanes (C_8-C_{20}) in order of elution on HP-5MS column. nd = not detected; SD: standard deviation (*n*=3).; PCM: Protected cultivation and morning harvest; PCA: Protected cultivation and afternoon harvest; FCM: Field cultivation and morning harvest; FCA: Field cultivation and afternoon harvest; Mix: Equivolumetric blend of the all distilled essential oils.

The chemical composition of *P. amboinicus* EO has been reported previously by several authors (Bandeira *et al*., 2011; El-Hawary *et al*., 2013; Murthy, Ramalakshmi, Srinivas, 2009). However, comparing those reported data with those of the present study, a huge difference in the qualitative and quantitative composition of these oils could be observed. The most similar chemical composition to our results was reported by Murthy, Ramalakshmi, Srinivas (2009). These authors achieved 70% carvacrol content, and more than 50% of the identified chemical constituents matched those of our identified chemical compounds. The other previously mentioned studies on carvacrol content reported a range from 0.00 to 19.29% and an expressive difference in qualitative chemical composition (Bandeira *et al*., 2011; El-Hawary *et al*., 2013).

The antioxidant activities of the *P. amboinicus* EO changed according to the type of cultivation and harvest time (Table III). The total antioxidant capacity (TAC) varied from 1548.1447 to 1904.7620 mg AAE/g (Table III). However, the EO of *P. amboinicus* showed very potent antioxidant activity, independent of the growing and harvesting conditions. The highest TAC (1904.7620 mg AAE/g) was obtained from EO of plants grown under protected cultivation and harvested in the morning. This result may be associated with the higher levels of carvacrol (75.21%) and β-caryophyllene (7.70%) in this sample than in the other samples. Both carvacrol and β-caryophyllene have been reported in several studies that corroborate a variety of biological actions, such as anti-inflammatory, antioxidant, antibacterial, antifungal, hepatoprotective, and vasorelaxant activities (Chizzola, Michitsch, Franz, 2008).

Regarding the DPPH free radical scavenging ability, the EO of *P. amboinicus* demonstrated a lower activity compared to that of the positive control (BHT, IC_{50} = 0.1826 ± 0.0136 mg/mL). However, the antioxidant properties of the EO (0.36 to 0.51 mg/mL) should not be disregarded (Table III). Bezerra *et al.* (2017) also reported that *P. amboinicus* EO exhibited significant inhibition of DPPH free radicals.

TABLE III - Antioxidant activities and α-amylase and lipoxygenase enzymes inhibition of the essential oil of *P. amboinicus* distilled from leaves of plants growing and harvesting in different conditions

Harvest time	Field cultivation	Protected cultivation
TAC (mg AAE /g)		
Morning	1591.7077±1.5612 bA	1904.7620±6.5203 aA
Afternoon	1548.1447±2.0816 bB	1568.5744±1.3768 aB
DPPH (IC_{50}=mg/mL)		
Morning	0.4067±0.0153 aB	0.3633±0.0153 bB
Afternoon	0.5067±0.0153 aA	0.4700±0.0200 bA
ABTS (IC_{50}=mg/mL)		
Morning	0.0067±0.0001 aB	0.0058±0.0001 bB
Afternoon	0.0078±0.0001 aA	0.0077±0.0001 aA
α-amylase (IC_{50}=mg/mL)		
Morning	0.6433±0.0115 aB	0.6267±0.0208 bB
Afternoon	0.6867±0.0058 aA	0.6600±0.0200 bA
Lipoxygenase (IC_{50}=mg/mL)		
Morning	1.5467±0.0493 aB	1.4967±0.0208 bB
Afternoon	1.5767±0.0115 aA	1.5567±0.0153 bA

The free radical ABTS scavenging method supported the antioxidant activity of *P. amboinicus* EO. The ABTS inhibitory concentration showed IC_{50} values ranging from 0.0058 to 0.0078 mg/mL. The antioxidant activity of the EO distilled from plants grown in protected cultivation conditions that were harvested in the morning (0.0058 mg/mL) was 1.27 times greater than that of the positive control used in the ABTS assay (Trolox, IC_{50} = 0.0074

± 0.0001 mg/mL). In addition, Romano *et al*. (2009) reported that phytochemicals may have distinct kinetic behavior for capturing free radicals. Thus, this could explain the differences observed in the DPPH and ABTS assays for EOs of *P. amboinicus*. The antioxidant activities of the EOs of *P. amboinicus* could be related to the high content of carvacrol (68.92% to 75.21%), since several studies have reported the antioxidant activity of carvacrol (Bezerra *et al*., 2017; Chizzola, Michitsch, Franz, 2008).

For the first time, in the present study, the potential of *P. amboinicus* EO on the enzymatic inhibition of α-amylase and lipoxygenase was evaluated (Table III). Inhibition of α-amylase and lipoxygenase enzymes provides a biochemical basis for the management of type 2 diabetes by controlling glucose absorption and reducing inflammation, respectively (Kirakosyan *et al*., 2018). Independent of growing and harvesting conditions, the studied concentrations of the EO *P. amboinicus* leaves did not show α-amylase and lipoxygenase enzymatic inhibition compared to that of the positive controls. The IC_{50} of the *P. amboinicus* EO on α-amylase (0.6267 to 0.6867 mg/mL) and lipoxygenase (1.4967 to 1.5767 mg/mL) enzyme inhibition were approximately 100 and 80 times lower, respectively, than those of the phaseolamine (0.0067 mg/mL) and NDGA (0.0200 mg/mL) positive controls.

Our findings indicate that *P. amboinicus* EO, under the conditions used, does not inhibit α-amylase and lipoxygenase enzymes, possibly not acting on the control of diabetes and/or its complications. However, the significant antioxidant activity of *P. amboinicus* EO, probably due to its high carvacrol content, corroborates the reduction of oxidative stress and inflammation conditions that are present in diabetes. Some evidence of the protective effects of carvacrol on symptoms of diabetes has been reported (Bayramoglu *et al*., 2014; Ezhumalai, Radhiga, Pugalendi, 2014). Bayramoglu *et al*. (2014) demonstrated that the oral administration of 25 and 50 mg/kg body weight carvacrol to diabetic rats for 7 days resulted in a slight reduction in serum glucose levels; however, carvacrol has at least a partially protective role on liver enzymes. Ezhumalai, Radhiga, Pugalendi (2014) also provided similar results for the oral administration of carvacrol in combination with rosiglitazone since hepatic marker enzymes, such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gammaglutamyl transpeptidase, increased in high-fat diet-induced type 2 diabetic C57BL/6J male mice.

Another aspect of diabetic metabolic disorders is related to the predisposition of patients to fungal infections, including those related to *Candida* spp., due to an immunosuppressive effect (Rodrigues, Rodrigues, Henriques, 2019). For this reason, we evaluated a blend of all distilled *P. amboinicus* EO against eleven strains of *Candida*. The blended EO of *P. amboinicus* comprised 71.04% carvacrol. The anti-*Candida* sensitivity of *P. amboinicus* EO depends on the targeted yeast of *Candida* (Table IV). *C. albicans* ATCC 90028 and *C. dubliniensis* were the most sensitive *Candida* strains to the essential, with the highest mean zones of inhibition (42.0364 ± 0.0023 and 40.0553 ± 0.0049 mm, respectively). *C. kruseii* showed lower susceptibility to *P. amboinicus* EO than that of the abovementioned strains (28.0125± 0.0007 mm). The other selected *Candida* spp. showed inhibition zones ranging from 31.0976 ± 0.0051 to 38.0905 ± 0.0031 mm.

TABLE IV - Inhibition zone of essential oil of *P. amboinicus* against selected strain of *Candida*

Microorganisms	Inhibition zone (mm)
Candida rugosa IZ-12	32.0429 ± 0.0031
Candida kruseii ATCC 6258	28.0125 ± 0.0007
Candida tropicalis CBS 94	31.0976 ± 0.0051
Candida dubliniensis CBS 7987	40.0553 ± 0.0049
Candida albicans ATCC 90028	42.0364 ± 0.0023
Candida utilis CBS 5609	38.0889 ± 0.0046
Candida kruseii CBS 572	38.0319 ± 0.0029
Candida lusitanea IZ-06	36.0497 ± 0.0057
Candida glabrata IZ-07	37.0141 ± 0.0004
Candida glabrata ATCC 5207	38.0905 ± 0.0031
Candida albicans CBS 562	35.0574 ± 0.0048

The data represents the mean ± standard deviation (n=3).

Some authors have related carvacrol with the antimicrobial properties of EO of diverse Lamiaceae species and therefore regard it as an active compound (Cid-Perez *et al*., 2019; Rodrigues, Rodrigues, Henriques, 2019). The relatively high carvacrol content in *P. amboinicus* EO was unable to exhibit the expected anti-*Candida* inhibition. According to Rodrigues, Rodrigues and Henriques (2019), *C. albicans* is one the most significant *Candida* spp. causing onychomycosis, and it is known that patients with diabetes have a high rate of tinea pedis and onychomycosis; this infection is considered to be a predictor of diabetic foot syndrome.

In a recent study, Kumar *et al*. (2016) indicated that the percentage of prevalence of different *Candida* spp. causes diabetic foot ulcers as follows: *C. tropicalis* (34.6%), *C. albicans* (29.3%), *C. krusei* (16.0%), *C. parapsilosis* (10.6%), and *C. glabrata* (9.33%). Except for *C. parapsilosis*, the EO of *P. amboinicus* showed efficient growth inhibition of the causal *Candida* strains on diabetic foot ulcers. However, further investigations should be performed to validate the use of this EO as an herbal ingredient in antifungal formulations.

CONCLUSIONS

The extraction method, the growing and harvest time conditions highly influence the chemical composition and biological activities of *P. amboinicus*, *P. ornatus* and *P. barbatus*. The presence of non-volatile and volatile bioactive compounds greatly contributed to the antioxidant and anti-*Candida* activities of their extracts. Independent of *Plectranthus* species, extracts produced via alcoholic turbo-extraction or aqueous refluxing had similar antioxidant activities. However, the *P. barbatus* extract obtained via alcoholic turbo-extraction was the most promising antioxidant extract. Furthermore, *P. amboinicus* EO has significant antioxidant and anti-*Candida* activities.

Our results clearly indicate that extracts prepared from *Plectranthus amboinicus*, *P. ornatus* and *P. barbatus* or *P. amboinicus* EO can find practical applications in the management of diabetes and/or its complications. However, an in-depth biochemical investigation of these three underexplored *Plectranthus* species is mandatory to identify novel herbal preparations and valuable metabolites for therapeutic purposes in the pathogenesis of diabetes-related conditions.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest regarding the submitted research article.

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